# FACTORS AFFECTING THE LEVEL OF INFECTIVITY OF RNA OF VENEQUELAN EQUINE ENCEPHALOMYELITIS (VEE) VIRUS

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In this work, RNA of VEE virus isolated from virions and from infected cells was studied. The main parameters for detection of maximum activity of preparations of viral RNAs were determined. It was shown that among the factors determining the level of RNA infectivity considerable importance belonged to the time of RNA adsorption on cells, concentration and duration of treatment of cells with NaCl as well as addition to the agar overlay of polycations (DEAE-dextran, protamine sulfate). Stability of RNA preparations upon storage and their thermostability were studied. All the data obtained permit to recommend optimal conditions for detection of infectious activity of VEE virus RNA.





## FACTORS AFFECTING THE INFECTIVITY LEVEL OF RNA OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS

[Paper by A. S. Agabalyan, L. K. Mer'shikh and F. I. Yershov, Institute of Virology imeni D. I. Ivanovskiy, USSR Academy of Medical Sciences, Moscow; Voprosy Virusologii (Problems of Virology), No. 5 (?)1971, pp. 527-532. Received by editors 13 July 1970].

[RNA of the Venezuelan equine encephalomyelitis (VEE) virus, isolated from virions and infected cells was studied, and the main parameters for detection of maximal infectivity of the viral RNA were determined. It was shown that, among the factors determining the RNA infectivity level, adsorption time of the RNA on cells, the concentration and duration of treatment of the cells with NaCl, and the addition of polycations (DEAE-dextran, protamine sulfate) to the agar overlay, are all of significant importance. The data obtained in the study suggest optimal conditions for determining the infectious activity of the RNA of the VEE virus].

Up to now, infectious RNA's have been isolated from many different RNA viruses. These include a number of the Group A arboviruses (eastern and western equine encephalomyelitis, Semliki* wood, Sindbis* and Chikungunya viruses [3, 6, 10, 14, 15]). In the studies referred to, a number of different methods were used to detect and determine the infectious properties of RNA, and this diversity of technique explains the inconsistency of the results obtained. In the case of the arboviruses, infectious RNA was obtained from the brains of infected mice, infected cells, and virion suspensions [3, 15]. The phenol deproteinization method, in a great variety of modifications, is the one most widely used to isolate RNA. In a few studies RNA has been detected with the use of detergents [2].

In the early studies, titration of the infectivity of viral RNA's was

*Transliterated from the Russian.

realized with the use of intracerebral administration of preparations to newborn mice [3]. Very soon, however, a more precise and sensitive method of titrating RNA infectivity was devised, using the plaques formed under the agar overlay [1, 13] of cell cultures. Improvement of this method followed, along the line of increasing titers of infectious RNA. Here, as in the extraction process, various additives were used--bentonite, sodium desoxylite, sodium dodecylsulfate, DEAE-dextran, poliornityl, dimethyl sulfoxide, protamine sulfate, and some others. These additives favored an increase in cell permeability, or protected the RNA from the distructive action of ribonuclease.

Optimal conditions for observing the infectious properties of RNA vary widely, depending on the properties of the Venezuelan equine encephalomyelitis (VEE) virus, the form of the cell culture used, and the conditions of culturing.

The aim of the present study was to determine optimal conditions for making clear the infectious properties of the RNA of the VEE virus, a representative of the arbovirus Group A.

Materials and methods. The virus. The VEE virus used was obtained from the Museum of Virus Strains, Institute of Virology imeni Ivanovskiy, USSR Academy of Medical Sciences. The virus went through up to 30 laboratory passages on chicken embryo fibroblasts. The virus-containing liquid obtained 18 hours following infection of the monolayer served as the source of RNA, as did the infected cells themselves. The viruses were titrated with use of the plaque-under-agar-overlay method [11].

Cells. Chicken fibroblasts, obtained with trypsinization of 10-11-day-old embryos, were cultured in a mixture of lactalbumin hydrosate and Medium No. 199 (1:1) with 10% beef serum, for 2-3 days before formation of the monolayer.

Isolation of RNA. The infectious RNA was isolated by the phenol-detergent method. The extraction process was as follows: to the virus-containing suspension was added 0.5% of sodium dodecylsulfate and an equal volume of freshly distilled phenol, heated to 65°C and saturated with tris-HCl with a buffer. The mixture was agitated for 5-7 minutes at 65°, then centrifuged at 5,000 rpm for 20 min. The surperfluous liquid was removed, and the extraction repeated, this time without the addition of detergent. The aqueous phase, following a second centrifuging, was treated 5-6 times with a double volume of distilled ether to remove the remnants of phenol. The ether was removed from the solution by nitrogen blow-through, and the RNA was precipitated by a 2.5-volume of 96% ethanol.

RNA from the infected cells was isolated by the same method, but with use of cold phenol.

Determination of the infectious properties of the RNA. The method of plaques under agar overlay was used for the titrimetry of RNA

RNA infectivity. Dilutions of the RNA were prepared on 0.1 M tris-HCl buffer with 1 M NaCl, pH 7.4-7.6. The cells, before infection, were treated with the same buffer for 15 min. at room temperature. To each culture was added 0.2 ml of the corresponding RNA dilution. Adsorption of the latter on the cells was continued for 2-5 min. at room temperature. Unadsorbed RNA was removed, following which the monolayer was covered with an agar overlay. After 48 hrs. of incubation, the monolayer was dyed and the number of plaques determined.

In the control tests, the RNA was treated with ribonuclease (Worthington) and with serum immune to VEE virus. To obtain the latter, rabbits were injected intravenously 3-4 times with 1 ml of virus-containing suspension ($10 \cdot 10^9$ BVU/ml) at weekly intervals. The last injection was both intravenous and intra-abdominal. Serum titer in the hemaglutination-inhibition reaction was 1:10,000, and in the neutralization reaction 1:33,000.

Reagents. DEAE-dextran ("Sigma", U.S.A.), protamine sulfate ("Spofa", Czechoslovakia), trypsin ("Difco", U.S.A.).

TABLE 1

Effect of Processing Time and NaCl Concentration on Titer of Infectious RNA

| NaCl concentration (M) | Titer of infectious RNA (in log BVU/ml) | | | | |
|---|---|---|---|---|---|
| | Processing time (min.) | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| 0.5 | 1.6 | 1.7 | 2.0 | 1.9 | 1.1 |
| 1.0 | 2.3 | 2.8 | 3.5 | 2.3 | 1.7 |
| 1.5 | 2.4 | 2.3 | 1.5 | 0.0 | 0.0 |
| 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

NOTE: Zero entries denote the absence of plaques, resulting from destruction of cells.

## Results

1. The effect of processing time and NaCl on infectious RNA titer. It was established in a series of tests that the usual method of virus titrimetry is unsuitable for determining the infectious activity of RNA preparations. To

assure permeability of the cells to RNA, the monolayer was treated with various concentrations of NaCl. It was observed that maximal titers of RNA infectivity appeared following treatment of the cells with 1 M NaCl solution for 15 min. at room temperature. As is evident from Table 1 above, increase in concentration, just as prolongation of cell-NaCl contact, as a rule results in a perceptible reduction in the number of plaques. Reduction of infectious RNA titer is observed also in connection with use of a hypotonic NaCl solution, or reduction in processing time.

2. The Effect of DEAE-dextran and protamine sulfate. As is well known, polycations of the type of DEAE-dextran, protamine sulfate and poliornithine have an effect on the number and size of plaques formed by infectious RNA under an agar overlay [1, 2, 7, 12, 13].

In the studies cited, polycations were used both in treating the cells and for thinning the RNA; in some cases, too, they formed part of the composition of the agar overlay.

In our own tests, the effect of various concentrations of DEAE-dextran on plaque-formation caused by infectious RNA was determined. It was established that the highest degree of infectious activity results from the addition of 2-3 mg/ml of DEAE-dextran (Figure 1). The introduction of the indicated concentrations of polycation led to an increase in the number of plaques. The plaque diameter increased perceptibly, reaching 5-6 mm in a number of cases (Figure 2).

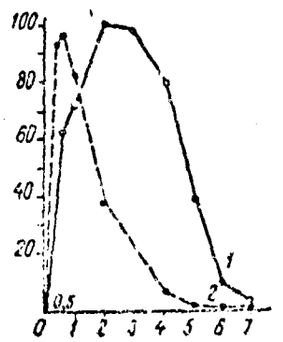


Figure 1. Effect of various concentrations of DEAE-dextran and protamine sulfate on infectious RNA titer.

The DEAE-dextramine and protamine sulfate in various concentrations were added to the agar overlay. Maximal values of the RNA are taken as 100%.

1 - DEAE-dextran; 2 - protamine sulfate. RNA titer (in %) is plotted on the y-axis; concentration of DEAE-dextran and protamine sulfate (in mg/ml) on the x-axis.

Figure 2. Plaques formed by infectious RNA of VEE virus. On the monolayer was imposed 0.2 ml of material in a dilution of $10^{-1}$. After 2 hrs. of incubation at 37°C, the monolayer was dyed and the number of plaques counted.

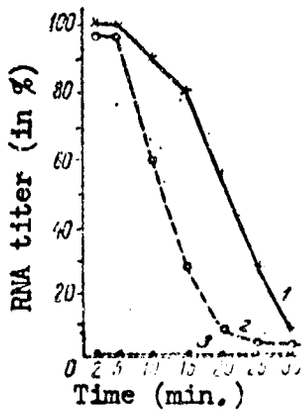


Figure 3. Conditions of adsorption of infectious RNA on cells.

Adsorption was produced at various time intervals and at various temperatures. Values of RNA obtained with 5-minute adsorption were taken as 100%. 1 - room temperature; 2 - 37°C; 3 - 4°C.

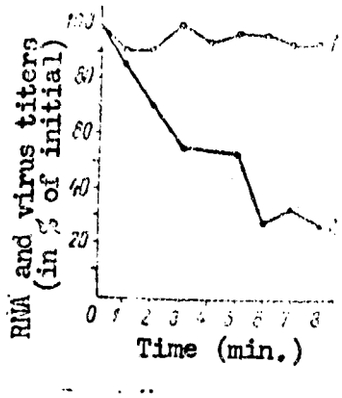


Figure 4. Sensitivity of infectious RNA and VEE virus to heating.

Preparations of virus and RNA were heated at 56°C for various periods.

1 - infectious RNA; 2 - initial virus

At the same time, optimal concentration of protamine sulfate, introduced into the agar overlay in place of DEAE-dextran, was determined. It was found that with use of 0.3-0.6 mg/ml protamine sulfate, titers of infectious RNA approximated the maximal values (Figure 1); however, in this case the plaques were of smaller diameter, and less distinctly shaped, than with use of DEAE-dextran. The use of high concentrations of DEAE-dextran and protamine sulfate, with the optimal values indicated above, led to a marked lowering of the infectivity titers of the RNA.

3. Adsorption of infectious RNA. As distinct from viruses, which require 30-60 min. for adsorption on cells, RNA is absorbed a great deal more rapidly. We were able to establish in a series of experiments that the highest RNA titers are found following adsorption of the RNA in the course of 2-3 min. at room temperature, and also at 37°C. In the case of adsorption of RNA at 4°C, no plaque-formation takes place (Figure 3). As is evident from Figure 3, when adsorption time is increased beyond the optimal length, there is a marked drop in infectivity titers.

4. Action of ribonuclease, trypsin and immune serum. In a series of tests we determined the action of enzymes and immune serum on preparations of infectious RNA (Table 2). The initial virus was used as a control. It was demonstrated that treatment with ribonuclease completely inactivates the infectious properties of the RNA, whereas treating the initial virus with ribonuclease produces only a negligible reduction in infectious capability, or none at all.

TABLE 2

Effect of Various Factors on the Infectivity of VEE Virus and RNA Preparations

| Type of treatment | Infectivity titers (in log BVU/ml) | | | | | |
|---|---|---|---|---|---|---|
| | RNA | | | Initial virus | | |
| | Before treatment | After treatment | Log of inhibition (—) or increase (+) | Before treatment | After treatment | Log of inhibition (—) or increase (+) |
| Ribonuclease[1] | 3.0 | 0 | —3.0 | 8.1 | 8.0 | 0 |
| Trypsin[2] | 3.0 | 3.0 | 0 | 7.8 | 7.8 | —0.3 |
| Immune serum[3] | 3.1 | 2.9 | —0.2 | 6.0 | 6.0 | —2.1 |
| 1 M NaCl[4] | 0 | 3.5 | +3.5 | 7.1 | 7.1 | —1.7 |
| DEAE-dextran[5] | 0 | 3.5 | +3.5 | 9.0 | 9.0 | +0.2 |
| Protamine sulfate[5] | 0 | 3.4 | +3.4 | 8.9 | 8.9 | +0.1 |

[1]RNA and virus preparations treated with ribonuclease (5 μg/ml) for 30 min. at room temperature.

[2]RNA and initial virus treated with trypsin (5 μg/ml) for 30 min. at room temperature.

[3]RNA and virus preparations treated with serum (1:100, titer 1:33,000) for 1 hr. at room temperature.

[4]Monolayer of cells treated with 1 M NaCl at room temperature for 15 min. before addition of virus.

[5]In optimal concentrations.

The serum immune to VEE virus had no effect on plaque-formation produced by RNA preparations; however, it significantly lowered the infectious titers of the initial virus.

We should recall, also, the toxic effect which the neutral red dye, usually added to reveal plaques in the agar overlay, has on the infectivity of the RNA preparations. The the number of plaques is usually reduced by a factor of several times, in comparison with those found in the dyed monolayer after two days of incubation.

5. Sensitivity of the initial virus and the RNA preparations to heating. Heat-resistance is a characteristic feature of infectious RNA. With incubation for various periods of time at 56°C, infectivity of the substance remains quite unchanged, whereas similar heating of the initial virus leads to protein coagulation, thus producing inactivation of the virus in 2-3 minutes of heating (Figure 4).

6. Stability of infectious RNA's isolated from various sources, during the process of storage. Infectious RNA isolated either from virus suspensions or from infected cells preserves its activity best when stored at —20°C. At —10°C, however, it will retain its potency for 7-10 days. As is evident from Table 3, RNA isolated from a virus suspension is significantly more stable (preserving its infectivity for 4-5 weeks) than RNA obtained from infected cells, which begins to become inactivated by the end of the second week of storage.

TABLE 3

Stability of Infectious RNA's of VEE Virus, in Storage*

| Infectious R N A | Infectious RNA titer (in log BVU/ml) | | | | |
|---|---|---|---|---|---|
| | Storage period (weeks) | | | | |
| | 1 | 2 | 3 | 4 | 5 |
| From virus suspension | 4.0 | 3.7 | 3.5 | 3.5 | 2.8 |
| From infected cells | 3.0 | 1.8 | 0.7 | 0 | 0 |

*Preparations stored at —20°C.

## Discussion

RNA of VEE virus can be isolated from various sources with the phenol-detergent method. The RNA preparations thus obtained have an infrared absorption spectrum which is characteristic of the nucleic acids. The data of tests made to determine RNA sensitivity to various enzymes and to immune serum (See Table 2) indicate that the infectivity of preparations obtained as a result of phenol deproteinazation of viral particles is that of virus RNA and not that of a residual virus.

The process of adsorption of RNA by cells is an extremely rapid one which is evidently complete after 5 min. of contact. The subsequent drop in RNA

infectivity level which we observed may be associated both with the destructive action of ribonuclease and with the cytodestructive action of the 1 M NaCl on the cells.

Most researchers emphasize the necessity of using hypertonic solutions in the process of titrating infectious RNA. Concentration of the salt and time of application to the cells depend first of all upon the type of tissue culture [5] used. Igarashi et al. [6], for example, in order to obtain maximal titers treated cells with Vero 2 M $MgSO_4$, in contrast to our own experiments, in which the optimal concentration was found to be 1 M (NaCl).

Polycations used to raise the infectivity of RNA may act in various ways, interacting both with the cells and with the RNA itself. In the first instance they raise the cell permeability to the nucleic acids; in the second, the RNA-polycation complex which is formed may, possibly, effectively protect the nucleic acid from the action of ribonuclease.

The use of optimal concentrations of DEAE-dextran, protamine sulfate, and a number of other polycations has enabled many researchers to achieve the very best results in determining the infectious properties of RNA viruses [1, 2, 4, 7, 8, 9, 12]. Here it should be emphasized that reduction in infection titers, which is observed with increase in the concentration of the polycations above the optimal, may possibly be the result of the toxic action of the polycations on the cells.

As indicated by the data shown in Table 2, this conclusion is justified for the RNA of the VEE virus.

One should emphasize, in particular, the heat-resistance of nucleic acid. It was shown in our tests that deproteinized RNA is not inactivated by temperatures of 56°C, whereas the same temperature will inactivate the virus, probably as a result of coagulation of proteins, which on being displaced form conglomerates with the nucleic acid, thereby preventing the possibility of the latter exerting its infective capabilities.

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